

Proteomic Analysis of Human Neuronal Cells Treated with the Gulf War Agent Pyridostigmine Bromide

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Abstract

Gulf War Illness (GWI) is characterized by a wide array of symptomology, which is possibly linked to the prophylactic treatment with pyridostigmine bromide (PB) against neurotoxins. It is now hypothesized that the pathological origin of this multi-symptom illness lies within the central nervous system and is caused by irregular activation of neuronal signaling pathways. To investigate this possibility, a proteomic-based approach was applied to characterize cellular responses of neuronal cells to PB exposure. Protein extracts from cultured neuroblastoma cells treated with 700nM PB for 10 days, as well as extracts from control cells were separated using two-dimensional gel electrophoresis. Twenty two differentially-expressed proteins were identified by MALDI-TOF mass spectrometry (MS). Ingenuity Pathways Analysis (IPA) software was then used to determine the biological functions and canonical pathways associated with the PB-responsive proteins.

Keywords: Gulf war illness; Pyridostigmine bromide; Multi-symptom illness; Proteomics; Two-dimensional gel electrophoresis, MALDI-TOF MS

Abbreviations: PB: Pyridostigmine Bromide; MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry; GWI: Gulf War Illness; IPA: Ingenuity Pathway Analysis; 2DGE: Two-Dimensional Gel Electrophoresis; IEF: Isoelectric Focusing; hnRNP: heterogeneous nuclear Ribonucleoprotein

Introduction

Veterans from the 1990-1991 Persian Gulf War conflict exhibit between 26%-32% more chronic and complex health problems than non-deployed veterans or veterans who were deployed elsewhere (Fukada et al., 1998; Steele et al., 2000). The time course of illness and the pattern of symptoms unique to this deployment are now characterized as Gulf War Illness (GWI), which affects between 175,000 to 210,000 US veterans. Among the 37 persistent symptoms, the most commonly reported included fatigue, gastrointestinal problems, chronic and widespread pain, neurological, and musculoskeletal symptoms (Steele et al., 2000). Pyridostigmine bromide (PB), a reversible carbamate acetylcholinesterase inhibitor, is considered to have an excellent safety profile in treatment of myasthenia gravis (Aquiloni et al., 1983). While still under consideration for approval by the FDA as a protective measure against toxic effects of nerve agents, PB was widely administered to soldiers during the 1990-1991 Gulf War (Binns et al., 2008).

Studies have shown an association between PB use during the Gulf War and subsequent development of neurocognitive and neuroendocrine dysfunction (Binns et al., 2008). Previous findings suggest that stress-induced compromise of the blood-brain-barrier (BBB) may allow PB to cross the BBB and induce neuronal toxicity and cell death in the brain (Abdel-Rahman et al., 2004). Recent evidence suggests, however, that passage of PB to the central nervous system (CNS) is not required for its neurotoxic effects, and may be mediated by its interaction with the peripheral nervous system (Amourette et al., 2009). Collectively, these findings have given rise to the hypothesis that the underlying cause of this multi-symptom illness may lie within the CNS, possibly due to aberrant activation of neural pathways (Binns et al., 2008).

Proteomic profiling of biological samples has become routine for the identification of biological and molecular pathways altered in response to particular stimuli and for a global overview of the disease process. Here, a two dimensional gel electrophoresis (2DGE)-based proteomic approach was used to identify the proteomic changes that occur in a neuronal cell culture model following a low-concentration PB treatment. Software-assisted analysis was then used to determine the biological networks that may be impacted by these differentially-expressed proteins.

Material and Methods

Cell Culture

Human neuronal cells (SH-SY5Y) were cultured in 50:50 DMEM:F12 supplemented with 10% FBS (Invitrogen, Carlsbad, CA) in 225cm² Corning flasks at 37°C with 5% CO₂ until confluent, and differentiated by adding 10 μM retinoic acid. Treated cells were incubated with media containing 700pM PB (treatment) or regular media (control).

Five control and five treated flasks were used for this experiment. Culture media was exchanged daily for 10 days for both control and treated groups, with the treated media being supplemented with PB daily. As outlined above, specific data

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are not available on the neuronal availability of oral PB; furthermore, considerable inconsistencies are reported for PB dosing by troops during the conflict. Therefore our aim was to observe the effects of low PB dosing and so we selected a 700nM dose which is approximately 100-fold less than the 1-2 hour plasma concentrations of PB for a single 30mg dose in a 77 kg adult male (Marino et al., 1998). Lactate dehydrogenase (LDH) activity was measured using a cytotoxicity detection kit (Roche, Indianapolis, IN). Clarified SH-SY5Y media was mixed with the reaction mixture at a 1:1 ratio in a 96 well microtiter plate. The resulting solution was incubated at room temperature for 15 minutes and absorbance was measured at 500nm using a chemoluminescent reader (Bio-Tek).

Protein Extraction, 2DGE, and Image Analyses

For protein extraction, the cells were first collected in PBS and centrifuged, and the pellet lysed by sonication in 3ml mammalian cell lysis buffer (M-PER, Pierce, Rockford, IL) supplemented with a protease inhibitor cocktail tablet (Calbiochem, San Diego, CA), followed by centrifugal clarification at 20,000 x g. The supernatant protein concentration was measured by the BCA assay (Pierce) according to the manufacturer's instructions. Two-dimensional gel electrophoresis (2DGE) experiments were performed using established methods (Poon et al., 2007). Briefly, 200 µg of protein extract from each flask was precipitated in four volumes of chilled acetone, re-suspended in isoelectric focusing (IEF) sample buffer that was supplemented with 0.5% Zoom Ampholytes (Invitrogen, Carlsbad, CA), and applied individually to 13 cm ReadyStrip™ IPG strips, pH 3-10, (Bio-Rad, Hercules, CA) for IEF. Second dimension separation was performed using linear gradient (8-16%) Precast Criterion Tris-HCl gels (Bio-Rad). Precision Protein™ Standard (Bio-Rad) was used to determine the relative molecular weight of each protein. The gels were stained in Flamingo stain (Bio-Rad) and imaged at 532nm using an FX Pro imager (Bio-Rad). A total of 5 gels per treatment group were used for statistical analyses and subsequent pooling of selected gel plugs.

PDQuest software (Bio-Rad) was used for matching gel displays, analyzing protein spots and determining the consensus gel image across all gels. The acquired gel images were normalized using the software to reduce any potential variation caused by uneven staining, and the spot intensities were calculated by integrating the optical density over the spot area. Spot intensities were compared between the two groups to determine differences in protein expression using the Student's t-test, with the α set at 0.05 to minimize type I error. Spots which were determined to be differentially-regulated by PDQuest were manually validated, and spots exhibiting poor gel-to-gel reproducibility within a given group (control or treatment) were discarded prior to MS peptide mass fingerprinting.

Protein Digestion and MALDI-TOF MS Analyses

Protein spots were excised using an ExQuest® spot cutter and digested according to Havlis and colleagues with minor modifications (Havlis et al., 2003). The selected gel pieces were destained in 50% acetonitrile (MeCN) for one week at -20°C and then dehydrated with 100% MeCN. The solvent

was removed by pipetting, followed by removal of residual liquid using a vacuum centrifuge. The gel pieces were re-suspended in 50µl of 10mM dithiothreitol, 50mM ammonium bicarbonate (ABC), and incubated at 50°C for 30 minutes. The samples were alkylated with 50mM iodoacetamide, 50mM ABC at 37°C for 45 minutes in the dark, and then washed for 10 minutes with 50% MeCN, dehydrated with 200µl of 100% MeCN and dried using a vacuum centrifuge. Each gel piece was then rehydrated with 2µl of 0.5µM trypsin solution at 4°C for one hour, followed by addition of 30µl of chilled 50mM ABC, and incubated at 56°C for 30 minutes. The digestion reaction was terminated by addition of 200µl of 50% MeCN and 1% formic acid, followed by incubation for one hour on a shaker at room temperature. The extracted peptides were transferred to a new tube and concentrated to approximately 5µL under vacuum, and further concentrated and de-salted in C-18 reverse phase Zip Tip columns (Millipore) followed by NuTip Hypercarb columns (Glygen) to capture remaining peptides not adsorbed by the C18 Zip Tips. Peptides were eluted from the columns using 2.5µL of 1mg/mL 4-alpha-cyano hydroxycinnamic acid (HCCA) in 50% MeCN, 25% methanol, and 0.1% trifluoroacetic acid, then spotted directly to a steel MALDI chip.

Mass spectra were acquired in positive ion reflectron mode in a MALDI-TOF-MS (Waters, MA). Peaks were obtained from the summed spectra of 20 combined spectra per sample. Each summed spectrum was smoothed, subtracted, and centroided prior to obtaining m/z values for peptide mass fingerprinting (PMF). Peaks derived from keratin or autocatalytic trypsin activity were manually subtracted from the peak list. Protein identities were determined by searching the peak lists against the SwissProt database (updated version dated 10/10/2007) using Protein Prospector v 4.0.7 MS-FIT (Clauser et al., 1999). Molecular weight, pI, and a mass tolerance of 25ppm were included as pre-filtering criteria. A static modification of +57.02 Da for carbamidomethyl cysteine, and a variable modification of +15.99 Da for oxidized methionine were included into the search parameters. Identifications were considered positive if the following three criteria were met: MOWSE score of > 100, at least 7 peptide matches and an agreement between observed and expected molecular weight and pI.

Ingenuity Pathway Analysis

A list of differentially-expressed proteins and their gene/protein ID numbers was uploaded to the Ingenuity Pathway Analysis (IPA) software to investigate the biological networks associated with these proteins (<http://www.ingenuity.com>). The IPA program uses a knowledgebase derived from the scientific literature to relate genes or proteins based on their interactions and functions. Ingenuity Pathway Analysis generates biological networks, canonical pathways and functions relevant to the uploaded dataset. Highly regulated biological networks and functions are identified using association rules among focus genes/proteins in a particular experiment. Each of these networks is scored and ranked for the enrichment of regulated genes/proteins in a particular network over competing networks. A right-tailed Fisher's exact test is used for calculating p-values to determine if the probability that the association

between the proteins in the dataset and the functional and canonical pathway can be explained by chance alone. The final scores are expressed as negative log of p-values and used for ranking.

Functional Annotation

DAVID bioinformatics "Functional Annotation Tool" was used to extract the biological meaning of differentially-expressed proteins. Briefly, the list of differentially-expressed

proteins was submitted using Uniprot ID designations as the identifiers. For functional categories, Swiss Prot Protein Information Resource Keywords (SP-PIR_KEYWORDS) were used. The resulting outputs were filtered to include only functionally-related identifiers (other identifiers such as subcellular location, etc., were excluded from this list). The data were then converted to a bar graph using Microsoft Excel.

Results

Proteome Response to PB Treatment

2DGE was used to separate crude protein extracts from SH-SY5Y cells grown in the presence or absence of 700pM PB. Figure 1 includes representative images of the 2DGE and MS based protein quantification and identification process. A consensus image was created for each using PDQuest software, from which differentially-expressed proteins could be identified. Initially, forty-nine proteins were determined to be differentially regulated, however following manual inspection, more than 50 percent of these were discarded due to poor reproducibility within a group. Twenty-one differentially-expressed proteins remained, and were identified using the filtering criteria as stated above in methods (Table 1 included as supplementary information), indicating that sub-nM concentrations of PB are sufficient to elicit proteomic changes in cultured nerve cells. These proteins consisted of enzymes such as peptidases and kinases, transcriptional regulators, transporters and ion channels. Though the stringency was set to a 1.5-fold differential regulation, there were some proteins that were down-regulated in treated cells by two-fold or higher including aldolase A, enolase 1, phosphoglycerate kinase 1, heterogenous

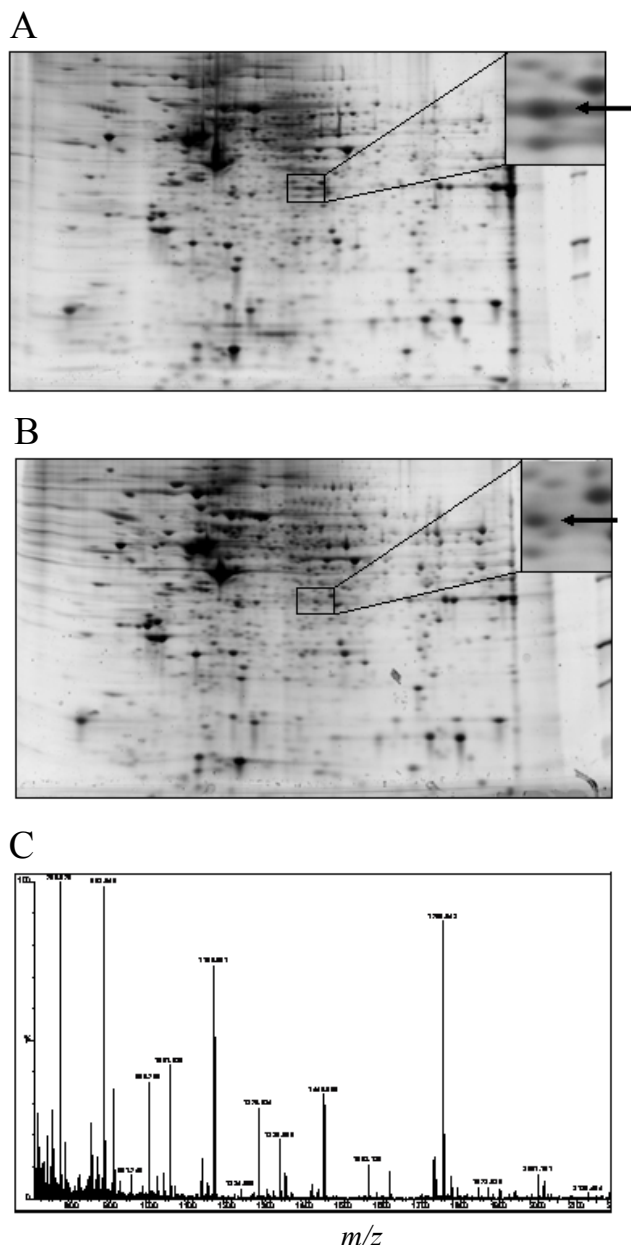


Figure 1: Representative 2D gels reveal differentially-expressed proteins between (A) PB-exposed and (B) control cultures after 10 days of treatment. 200 μ g of desalted protein was applied to pH 3-10 IPG strips for isoelectric focusing, and further separated on 8-16% tris-glycine SDS-PAGE gels. The gels were stained with a fluorescent dye and imaged. Differentially-expressed spots were cut from the gel, digested and (C) subject to MALDI-TOF MS analysis (see methods). Boxed in both is an expanded view of a ~35kDa protein (indicated by arrow) that was down-regulated following PB exposure for 10 days.

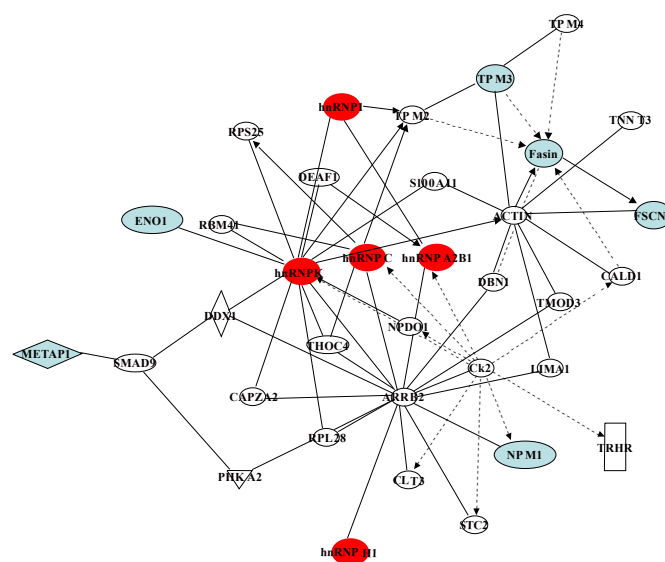


Figure 2: Software-assisted identification of putative biochemical networks affected by PB exposure, as revealed by Ingenuity Pathway Analysis (IPA). Differentially-expressed proteins showing a ± 1.5 -fold change or greater were selected and entered into IPA software to illustrate potential interactions. Solid arrows represent known physical interactions, dotted arrows represent indirect interactions. Red shapes indicate hnRNP protein family members identified by 2DGE in this study, blue shapes indicate other proteins in this network that were identified by 2DGE and mass spectrometric analysis.

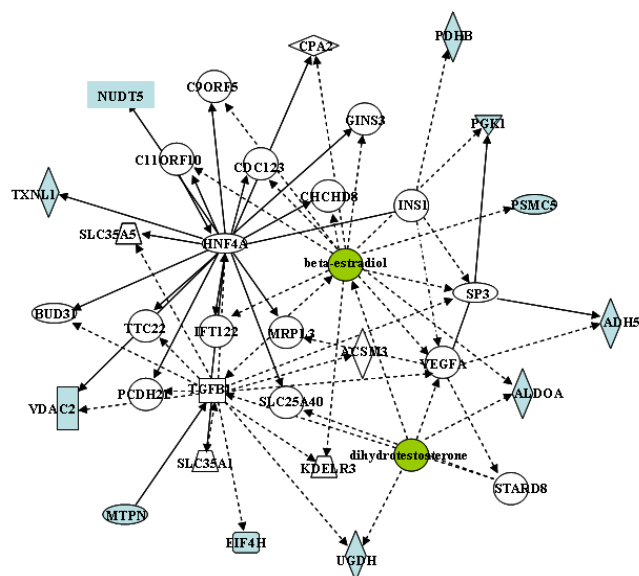


Figure 3: Software-assisted identification of putative biochemical networks affected by PB exposure, as revealed by Ingenuity Pathway Analysis (IPA). Differentially-expressed proteins showing a +/- 1.5-fold change or greater were selected and entered into IPA software to illustrate potential interactions. Solid arrows represent known physical interactions, dotted arrows represent indirect interactions. Green-shaded shapes indicate steroid hormones, while blue shapes indicate differentially-expressed proteins identified in this study.

nuclear ribonucleoprotein H1, methionyl aminopeptidase 1 and thioredoxin-like 1. All but one protein – tropomyosin alpha-3 chain – were down-regulated following prolonged exposure to PB.

Networks Induced by PB Treatment

Dynamic pathway modeling was performed using IPA software to examine the networks affected by exposure to PB. IPA segregated the list of differentially-expressed proteins into two non-overlapping networks and nearly-identical scores, one which included 10 of the regulated proteins, and the other which included 11 (Figures 2 and 3, respectively). Some of the functions associated with the 11-protein network included tissue development and gene expression, while functions associated with the 10-protein network included genetic disorder, and cellular assembly and organization. The various biochemical and cellular functions that were impacted by the 10-day low dose PB treatment are further summarized as a histogram (Figure 4).

Discussion

According to the current literature, this is the first *in vitro* proteomic study aimed at assessing the proteomic response of neuronal cells to PB exposure. Given the hypothesis that GWI pathogenesis may initiate in the CNS due to irregular activation of neuronal signaling pathways (Binns et al., 2008), the choice of a stable neuronal cell line (SH-SY5Y) in this proteomic study provides a suitable model for initial examination of the neuronal molecular changes consequent upon PB exposure, implicated as one of the causal factors in GWI.

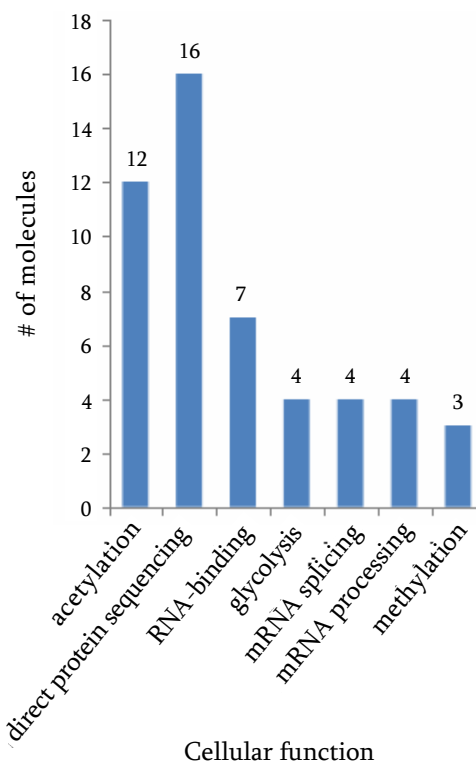


Figure 4: Histogram of differentially expressed proteins reveals the effects of PB exposure on multiple cellular functions.

Here, we demonstrated that significant biochemical changes can occur in neurons following exposure to PB at a concentration approximately two orders of magnitude below typical plasma PB concentrations following a 30 mg oral dose (Marino et al., 1998). The ability of PB to elicit expression changes in multiple proteins as presented here suggests that there are profound cellular responses to the drug, even at low concentrations. With such a low dose, even proteins exhibiting a +/- 1.5-fold change in protein expression as reported can be considered to be of potential pathogenic significance and worthy of further investigation in less limiting models of PB exposure such as animal models of GWI. A preliminary assessment of the potential functional significance of these protein changes, using the IPA knowledgebase, revealed that the regulated proteins could be functionally related and presented two potential networks of neuronal response to PB exposure, one of which (Figure 2) illustrated the involvement of several hnRNPs.

hnRNPs are a family of approximately 20 proteins (Pinol-Roma et al., 1988), and are among some of the most abundant RNA-binding proteins (He et al., 2008). These proteins are often highly abundant in a cell (Kiledjian et al., 1994) and are responsible for affecting cellular functions such as transcription, mRNA stability, pre-mRNA splicing, nuclear import and export, and cytoplasmic trafficking of mRNA (He et al., 2008). This coordinated down-regulation of several of these proteins following PB exposure may imply that the compound can impact one or more of the above-listed functions, even at such low concentrations. It is also possible that the observed effects of PB may not be specific to this particular drug and may be a

general class effect. Nonetheless, given the wide array of potential pathways that can be perturbed by alteration of RNA processing, such data warrants further validation and investigation into the role(s) of PB-elicited changes in hnRNP abundance. Inspection of the 11-protein network identified by IPA reveals several proteins that are peripheral to hormone action. This network was primarily associated with tissue development as well as gene expression. Subsequent studies will be undertaken to reveal any possible connection between the potential for gene regulation mediated by altered hnRNP protein expression and the effects of the proteins in the 11-protein network.

In an earlier study, using a microarray-based platform, we have evaluated the *in vitro* genomic effects of PB exposure in SH-SY5Y cells. The observed gene expression changes predicted several of the protein responses observed here (Kayihan et al., 2009, submitted). These proteins include aldolase A, eukaryotic translation initiation factor 4H, tropomyosin alpha-3, and thioredoxin-like protein 1. Similarly, several of the functional pathways identified here were also associated with PB exposure at the transcriptional level (Kayihan et al., 2009, submitted).

Future studies aimed at validating the signaling networks identified here using more advanced proteomic techniques such as SILAC (stable isotope labeling with amino acids in cell culture) or AQUA would allow for a more thorough interrogation of the cellular response to PB, in terms of number of proteins studied, as well as quantitative depth. Such an approach would also allow for examination of various subproteomes, such as organelle-specific extracts or post-translational modifications, with greater ease and precision than is possible with differential 2DGE analyses. The current study suffers from the acknowledged limitations of 2DGE technology, including limiting the evaluation to highly-abundant proteins between pI values of 3 and 10. Acidic and basic proteins whose pI values reside outside of this range are therefore eliminated from analysis, as are proteins with poor solubility that either do not enter, or do not focus, in an IEF slab gel. Expanding this research to animal models of exposure to Gulf War agents will provide validation for *in vitro* studies such as this one and offer many more opportunities for investigation and further insight into the pathology of GWI.

In summation, this study has demonstrated the use of 2DGE for examining the proteomic changes following low-dose PB exposure, and observed changes in several proteins which segregate into two potential networks. Further study will be required to determine if a causal relationship between PB exposure and GWI can be confirmed or refuted, and would ideally include more advanced approaches such as SILAC to more completely understand the response to exposure. *In vivo* rodent models of Gulf War agent exposure have been published by others and are in development in our laboratories, and the future proteomic analyses of those models will considerably advance our comprehension of the etiology of GWI. Nonetheless, in this limited *in vitro* study of the neuronal effects of PB our data suggest that at very low exposure this Gulf War agent can induce changes in protein expression which could be related to pathogenic consequences.

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